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The class V myosin motor protein, Myo2, plays a major role in mitochondrial motility in *Saccharomyces cerevisiae*

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The actin cytoskeleton is essential for polarized, bud-directed movement of cellular membranes in *Saccharomyces cerevisiae* and thus ensures accurate inheritance of organelles during cell division. Also, mitochondrial distribution and inheritance depend on the actin cytoskeleton, though the precise molecular mechanisms are unknown. Here, we establish the class V myosin motor protein, Myo2, as an important mediator of mitochondrial motility in budding yeast. We found that mutants with abnormal expression levels of Myo2 or its associated light chain, Mlc1, exhibit aberrant mitochondrial morphology and loss of mitochondrial DNA. Specific mutations in the globular tail of Myo2 lead to aggregation of mitochondria in the mother cell. Isolated mitochondria lacking functional Myo2 are severely impaired in their capacity to bind to actin filaments in vitro. Time-resolved fluorescence microscopy revealed a block of bud-directed anterograde mitochondrial movement in cargo binding–defective *myo2* mutant cells. We conclude that Myo2 plays an important and direct role for mitochondrial motility and inheritance in budding yeast.

Introduction

Mitochondria play a key role in cellular energy metabolism; they are the site of many anabolic and catabolic pathways, they are essential for the assembly of iron/sulfur clusters, and they participate in calcium signaling and regulation of cell death programs. Because of this multitude of cellular functions, the presence of mitochondria is essential for viability in virtually every eukaryotic cell. Mitochondria cannot be made de novo. Rather, they have to grow and divide from preexisting membranes, and their inheritance during cytokinesis involves ordered, cytoskeleton-dependent partitioning mechanisms (Nunnari and Walter, 1996; Warren and Wickner, 1996; Catlett and Weisman, 2000). Furthermore, cytoskeleton-based motility concomitant with frequent membrane fusion and fission is required to adapt mitochondrial copy number, morphology, and intracellular position to the cellular demands (Bereiter-Hahn, 1990; Yaffe, 1999; Chan, 2006; Dimmer and Scorrano, 2006). However, the molecular mechanisms governing mitochondrial motility, inheritance, and distribution within the cell are only poorly understood.

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Abbreviations used in this paper: DIC, differential interference contrast; Dox, doxycycline; mtDNA, mitochondrial DNA; mtGFP, mitochondria-targeted GFP.

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The budding yeast *Saccharomyces cerevisiae* has proven to be an excellent model organism to study the molecular basis of mitochondrial dynamics, and most of the evolutionarily conserved key components were first discovered in yeast (Hermann and Shaw, 1998; Jensen et al., 2000; Okamoto and Shaw, 2005; Merz et al., 2007). However, although many mechanistic details of the machineries mediating mitochondrial fusion and fission have been revealed in recent years (Shaw and Nunnari, 2002; Westermann, 2003; Hoppins et al., 2007), the identity of the proteins driving cytoskeleton-dependent mitochondrial movement in yeast is still under debate.

The evidence for a central role of the actin cytoskeleton in mitochondrial motility in budding yeast is clear cut. Numerous mutations in genes encoding actin or factors involved in actin filament dynamics lead to aberrant mitochondrial distribution and morphology (Drubin et al., 1993; Lazzarino et al., 1994; Simon et al., 1995; Smith et al., 1995; Hermann et al., 1997; Simon et al., 1997; Yang et al., 1999; Singer et al., 2000; Boldogh et al., 2001a; Fehrenbacher et al., 2004; Altmann and Westermann, 2005). Treatment of wild-type cells with actin filament–depolymerizing drugs produces defects in mitochondrial shape and movement (Boldogh et al., 1998), and fluorescence microscopy of fixed and live yeast cells revealed that mitochondria colocalize with

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and move along actin cables (Drubin et al., 1993; Lazzarino et al., 1994; Simon et al., 1997; Fehrenbacher et al., 2004). Isolated mitochondria bind to actin filaments in vitro in an ATP-sensitive manner (Lazzarino et al., 1994; Simon et al., 1995; Boldogh et al., 1998).

The myosin family of actin-based motors consists of at least 15 structurally and functionally distinct classes. In particular, class V family members have been shown to participate in numerous membrane trafficking events (Reck-Peterson et al., 2000). The yeast genome encodes five myosin-related motor proteins (Winsor and Schiebel, 1997). The *MYO1* gene encodes a class II myosin that, depending on the strain background, is either essential or nonessential for viability; the essential *MYO2* and the nonessential *MYO4* genes encode class V myosins and the nonessential *MYO3* and *MYO5* genes encode class I myosins. A myosin light chain that associates with Myo1 and Myo2 heavy chains is encoded by the essential *MLC1* gene (Stevens and Davis, 1998; Luo et al., 2004). In yeast, class V myosins are of major importance for polarized growth and actin-based organelle segregation. Cargos transported by Myo2 or Myo4 include post-Golgi secretory vesicles, the trans-Golgi network, the cortical endoplasmic reticulum, vacuolar membranes, peroxisomes, mRNA–protein complexes, and microtubule plus ends (Pruyne et al., 2004). Thus, it is tempting to speculate that mitochondria might also be transported by class V myosins. However, several genetic attempts to identify a myosin-like protein responsible for mitochondrial movement have failed so far. Δ*myo1*, Δ*myo3*, Δ*myo4*, and Δ*myo5* single deletion mutants (Simon et al., 1995; Dimmer et al., 2002; Boldogh et al., 2004) and a Δ*myo3* Δ*myo5* double mutant (Goodson et al., 1996) do not display major defects in mitochondrial distribution and morphology. Moreover, mitochondria have been reported to appear normal in certain conditional *myo2* mutants, namely *myo2-66* (Simon et al., 1995), *myo2-338* (Itoh et al., 2002), and *myo2-Δ6IQ* (Boldogh et al., 2004), and in double mutant *myo2-66* Δ*myo4* (Simon et al., 1995). Based on these findings, it is assumed that myosins do not play an important role in mitochondrial motility and inheritance in budding yeast (Boldogh et al., 2001b; Pruyne et al., 2004; Boldogh and Pon, 2006).

However, it has been reported that the *myo2-573* allele induces defects in mitochondrial distribution toward the bud (Itoh et al., 2002, 2004), and close inspection of mutants containing the *myo2-66* allele revealed an accumulation of mitochondria in the mother cell (Boldogh et al., 2004). Based on these results, a model has been proposed that assigns to Myo2 a rather indirect role in mitochondrial inheritance. According to this model, Myo2 drives movement of yet unknown retention factors from the mother cell to the bud tip, where these factors serve to anchor mitochondria to prevent retrograde movement back into the mother cell (Boldogh et al., 2004; Boldogh and Pon, 2006). In this scenario, entry of mitochondria into the bud would be mediated by myosin-independent mechanisms, and Myo2 would not be required for interaction of mitochondria with microfilaments.

We recently screened a collection of yeast strains containing essential genes under control of a titratable promoter to identify novel components involved in mitochondrial morphogenesis. The observation that depletion of Myo2 or Mlc1 results in the formation of highly aberrant mitochondria (Altmann and Westermann, 2005) prompted us to investigate whether Myo2 plays a direct role in mediating interactions of mitochondria with the cytoskeleton and bud-directed mitochondrial movement. Our results described here suggest that Myo2 is much more important for mitochondrial motility and inheritance than previously anticipated.

Results

Depletion of Myo2 induces defects in mitochondrial morphology

To investigate the role of essential myosin-related motor proteins in mitochondrial morphogenesis, we took advantage of promoter shutoff strains that carry the *MLC1*, *MYO1*, or *MYO2* gene under control of the $TetO_7$ promoter (Mnaimneh et al., 2004). Addition of doxycycline (Dox) to the medium efficiently represses the $TetO_7$ promoter, whereas promoter activity is high in the absence of Dox (Gari et al., 1997). Mitochondrial defects were never observed in $TetO_7$-*myo1* cells (Altmann and Westermann, 2005). In contrast, mitochondria have aberrant morphology in $TetO_7$-*mlc1* and $TetO_7$-*myo2* strains under repressive conditions but also in the absence of Dox (Altmann and Westermann, 2005). The latter conditions presumably result in nonphysiological overexpression of Myo2, which is expected to cause toxic effects because Mlc1 amounts become limiting (Stevens and Davis, 1998). Consistently, nonphysiological expression levels of Myo2 and Mlc1 lead to severe growth defects both in the absence and presence of Dox (Fig. 1 A).

To test the dependence of mitochondrial morphology on *MYO2* expression, we incubated $TetO_7$-*myo2* cells expressing mitochondria-targeted GFP (mtGFP) for different time periods in Dox-containing medium. Then, cells were fixed, microfilaments were stained with rhodamine phalloidin, and mitochondria and the actin cytoskeleton were visualized by fluorescence microscopy. Wild-type control cells displayed their characteristic tubular branched mitochondrial network, with actin patches concentrated in the bud and actin cables extending throughout the mother cell (Fig. 1 B). It should be mentioned that even extended growth on Dox-containing medium does not affect the actin cytoskeleton in wild-type cells (Altmann and Westermann, 2005). Already, in the absence of Dox, 35% of $TetO_7$-*myo2* cells contained misshapen, clumped, and often ring-shaped mitochondria in the presence of an apparently normal actin cytoskeleton (Fig. 1 B). This fraction of cells reached a maximum of 47% after 15 h of promoter repression. At later time points, an increasing fraction of cells contained a disorganized actin cytoskeleton in addition to altered mitochondria, presumably because of pleiotropic defects of Myo2 depletion. After 22 h of promoter repression, no cells with wild type–like mitochondria could be found (Fig. 1 B). Very similar results were obtained with the $TetO_7$-*mlc1* strain (Fig. 1 B). Interestingly, aberrant mitochondria could also be found in 36% of cells overexpressing *MYO2* from the *GAL* promoter compared with 4% in the control strain. We conclude that maintenance of mitochondrial morphology is sensitive to both overexpression and depletion of Myo2. The fact that a large fraction of cells contain aberrant

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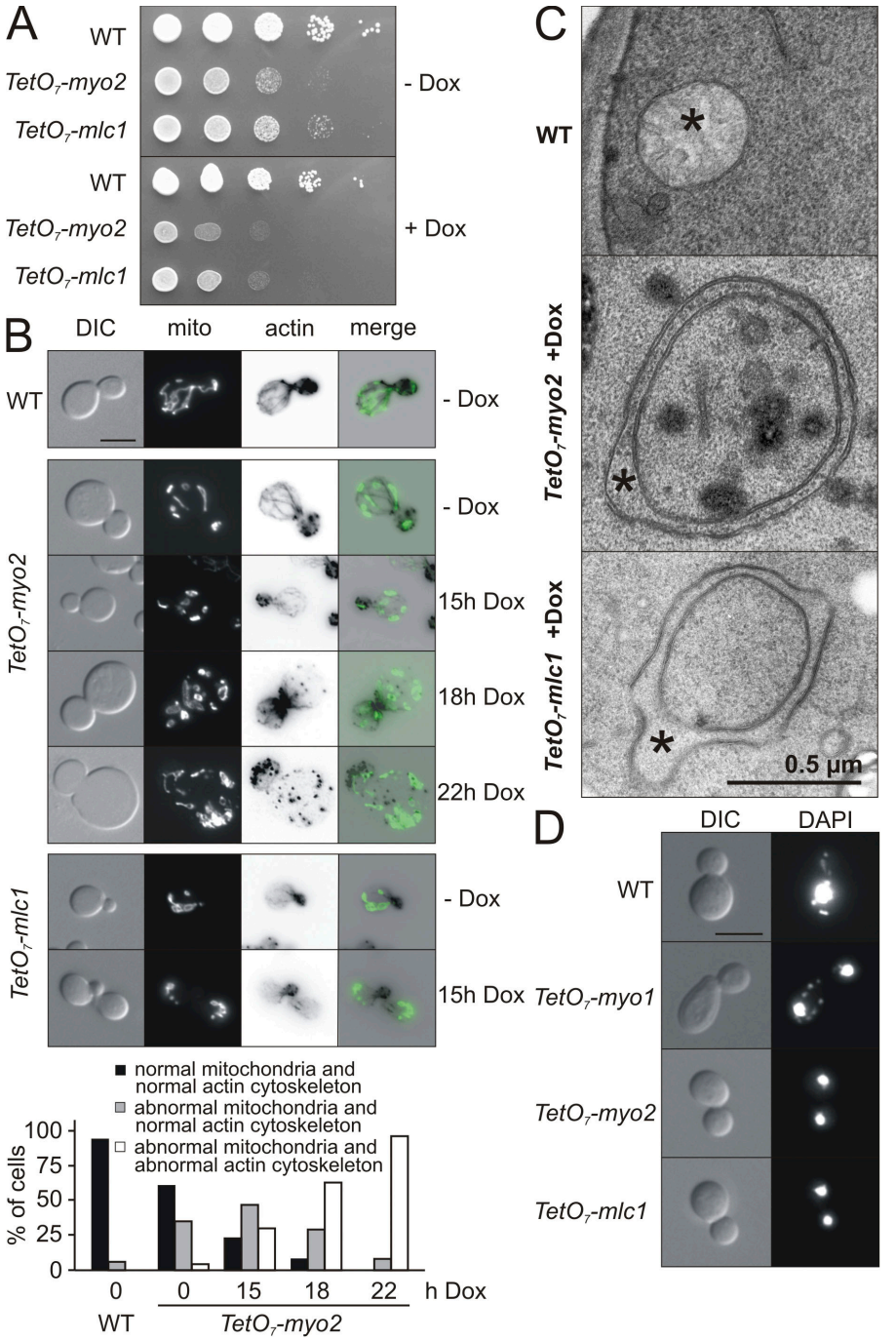

Figure 1. **Myo2 and Mlc1 are involved in mitochondrial morphology and inheritance.** (A) Serial dilutions of wild-type (WT), *TetO7-myo2*, and *TetO7-mlc1* cell suspensions were spotted onto plates with glucose-containing medium with or without 30 µg/ml Dox and incubated for three days at 30°C. (B) WT, *TetO7-myo2*, and *TetO7-mlc1* cells expressing mtGFP were grown for the indicated time periods in liquid glucose-containing medium in the absence or presence of 30 µg/ml Dox. Actin was stained with rhodamine phalloidin in fixed cells and cells were observed by fluorescence microscopy. (left) DIC image. (second from the left) Mitochondrial morphology (mito). (second from the right) Organization of filamentous actin (a reversed fluorescence image is shown to better visualize faint actin cables). (right) Merged image of reversed actin fluorescence and mitochondrial fluorescence. The graph at the bottom is a quantification of phenotypes of 100 cells per time point. Bar, 5 µm. (C) WT, *TetO7-myo2*, and *TetO7-mlc1* cells were grown to logarithmic growth phase in glucose-containing medium. *TetO7-myo2* and *TetO7-mlc1* cells were incubated in the presence of 30 µg/ml Dox for 15 h to deplete Myo2 or Mlc1. Ultrathin sections were analyzed by electron microscopy. All images are shown at the same magnification. Asterisks indicate the matrix space. (D) WT and *TetO7*-myosin strains were grown in glucose-containing medium, cellular DNA was stained with DAPI, and cells were analyzed by DIC and fluorescence microscopy. Nuclei are seen as large fluorescent spots; mtDNA nucleoids are seen as small fluorescent foci. Bar, 5 µm.

mitochondria in the presence of a normally organized actin cytoskeleton suggests that mitochondrial phenotypes seen in *TetO7-myo2* and *TetO7-mlc1* cells are not secondary consequences of cytoskeletal defects.

Next, we analyzed the ultrastructure of mitochondria in *TetO7-myo2* and *TetO7-mlc1* cells by electron microscopy. Promoter activity was repressed by growth in Dox-containing medium, cells were fixed and stained, and ultrathin sections were prepared and analyzed by transmission electron microscopy. Wild-type control cells displayed normal mitochondrial tubules with diameters of ~300–500 nm (Fig. 1 C). In contrast, cells depleted of Myo2 or Mlc1 frequently showed ring-shaped mitochondria with a very narrow matrix space of <100 nm in width (Fig. 1 C). This demonstrates that loss of Myo2 has severe effects on the internal structure of mitochondria. It is conceivable that the aberrant structures seen in electron micrographs correspond to the ring-shaped mitochondria observed by fluorescence microscopy (Fig. 1 B). Cross sections of complete yeast cells revealed an accumulation of coated vesicles in addition to aberrant mitochondrial morphology (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200709099/DC1), which suggests that secretion and bud growth are also impaired under these conditions.

We observed some residual growth of *TetO7-myo2* and *TetO7-mlc1* cells on Dox-containing medium (Fig. 1 A). This suggests

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that a basal expression of Myo2 and Mlc1 occurs even under repressive conditions. However, *$TetO_7$-myo2* and *$TetO_7$-mlc1* cells were unable to grow on nonfermentable carbon sources (unpublished data). As many mutants defective in mitochondrial morphology lose their mitochondrial genome (Berger and Yaffe, 2000), we asked whether respiratory deficiency of *$TetO_7$*-myosin strains might be caused by the loss of mitochondrial DNA (mtDNA). To test this, cellular DNA was visualized by DAPI staining and fluorescence microscopy in wild-type, *$TetO_7$-myo1*, *$TetO_7$-myo2*, and *$TetO_7$-mlc1* cells. Numerous spots of fluorescent mtDNA nucleoids were observed in wild-type and *$TetO_7$-myo1* cells but never in *$TetO_7$-myo2* and *$TetO_7$-mlc1* cells (Fig. 1 D). Thus, nonphysiologically high expression levels in the absence of Dox lead to the loss of the mitochondrial genome, which indicates that normal Myo2 and Mlc1 levels are required for maintenance of mtDNA. We conclude that Myo2 and its associated light chain Mlc1 are important for normal mitochondrial distribution and morphology, maintenance of the internal structure of mitochondria, and maintenance of mtDNA.

Myo2 and Mlc1 are required for binding of mitochondria to actin filaments in vitro

The interaction of isolated mitochondria with actin filaments in vitro has been shown to be ATP-sensitive, reversible, and dependent on mitochondria-associated proteins (Lazzarino et al., 1994). As these properties are compatible with the view that myosin-related motor proteins mediate organelle–cytoskeleton interactions, we asked whether Myo2 and Mlc1 are involved in this process. First, we incubated purified wild-type mitochondria with filamentous actin in the absence or presence of ATP, sedimented the organelles through a sucrose cushion, and detected bound actin by immunoblotting. Mitochondria were found to interact with actin filaments in an ATP-sensitive manner (Fig. 2 A, lanes 1 and 2; and Fig. 2 E). This binding activity could be removed by the extraction of mitochondria with high-salt buffer (Fig. 2 A, lanes 3 and 4; and Fig. 2 E) and was restored by the readdition of salt extract (Fig. 2 A, lanes 5 and 6; and Fig. 2 E). These results are very similar to observations made previously by Boldogh et al. (1998) and confirm that mitochondria–cytoskeleton interactions are mediated by proteins peripherally bound to the mitochondrial surface. Next, we tested mitochondria purified from *$TetO_7$-myo2* and *$TetO_7$-mlc1* cells grown under repressing conditions. Depletion of Myo2 reduced the mitochondrial actin binding capacity to 51% compared with the wild type (Fig. 2, B and E). Similarly, depletion of Mlc1 reduced actin binding activity to 55% (Fig. 2 B and E), demonstrating that Myo2 and its associated light chain are required for an efficient interaction of mitochondria with actin filaments. Remarkably, addition of salt extract prepared from wild-type mitochondria improved the actin binding activity of Myo2-depleted mitochondria from 51 to 95% (Fig. 2, C and E). This suggests that Myo2 is the peripheral mitochondria-associated factor that becomes limiting in *$TetO_7$-myo2* mitochondria. Moreover, incubation of wild-type mitochondria with affinity-purified antibodies directed against Myo2 abolished ATP-sensitive mitochondrial actin binding activity completely, whereas the same amount of antibodies directed against the mitochondrial inner membrane protein Mdm31 (Dimmer et al., 2005) had no effect (Fig. 2, D and E). This result demonstrates that Myo2 on the mitochondrial surface is required to establish interactions of the organelle with the cytoskeleton.

To corroborate these findings and observe the mitochondria–cytoskeleton interactions more directly, we used a visual in vitro assay. Mitochondria were isolated from mtGFP-expressing wild-type, *$TetO_7$-myo2*, and *$TetO_7$-mlc1* cells that were grown under repressive conditions. Purified mitochondria were incubated with Alexa Fluor 568–labeled actin filaments in the absence or presence of ATP and observed by fluorescence microscopy. In the absence of ATP, 91% of wild-type mitochondria were bound to actin. In contrast, only 45% of *$TetO_7$-myo2* and 51% of *$TetO_7$-mlc1* mitochondria were found in the vicinity of actin filaments (Fig. 2, F and G). In the presence of ATP, only 19–29% of mitochondria were found adjacent to actin filaments (Fig. 2 G), again indicating the ATP sensitivity of binding. Remarkably, only 16% of mitochondria pretreated with Myo2 antibodies were found next to actin filaments, whereas preincubation of mitochondria with Mdm31 antibodies as a control had no effect (Fig. 2, F and G). The fact that Myo2 antibodies blocked ATP-sensitive mitochondrial actin binding activity completely suggests that minor activity seen with Myo2-depleted mitochondria (Fig. 2, B, C, E, F, and G) is caused by residual expression of Myo2 in *$TetO_7$-myo2* cells under repressive conditions. We conclude from this series of experiments that Myo2 and its associated light chain are directly required for binding of mitochondria to the actin cytoskeleton.

Specific mutations in the Myo2 globular tail affect mitochondrial distribution and morphology

The carboxy terminal globular tail of Myo2 mediates specific binding of the myosin motor to cargo membranes such as vacuoles (Catlett and Weisman, 1998) and secretory vesicles (Schott et al., 1999). It consists of two structurally and functionally distinct subdomains; the proximal half binds to vacuolar membranes, whereas the distal half interacts with secretory vesicles (Catlett et al., 2000; Pashkova et al., 2005, 2006). We asked whether the Myo2 globular tail has a role in mitochondrial distribution and morphology. To test this, we used a series of point mutants carrying substitutions of single amino acid residues in regions critical for cargo binding. Alleles *myo2(Q1233R)*, *myo2(G1248D)*, *myo2(D1297N)*, *myo2(D1297G)*, *myo2(L1301P)*, *myo2(N1304S)*, *myo2(N1304D)*, and *myo2(N1307D)* cause specific defects in vacuolar inheritance (Catlett and Weisman, 1998; Catlett et al., 2000; Pashkova et al., 2006). These mutants do not show any growth defects, which indicates that essential cellular functions, such as secretion or organization of the cytoskeleton, are not severely impaired (Catlett and Weisman, 1998; Catlett et al., 2000; Pashkova et al., 2006). Alleles *myo2(L1331S)*, *myo2(L1411S)*, *myo2(Y1415E)*, *myo2(K1444A)*, and *myo2(Q1447R)* carry

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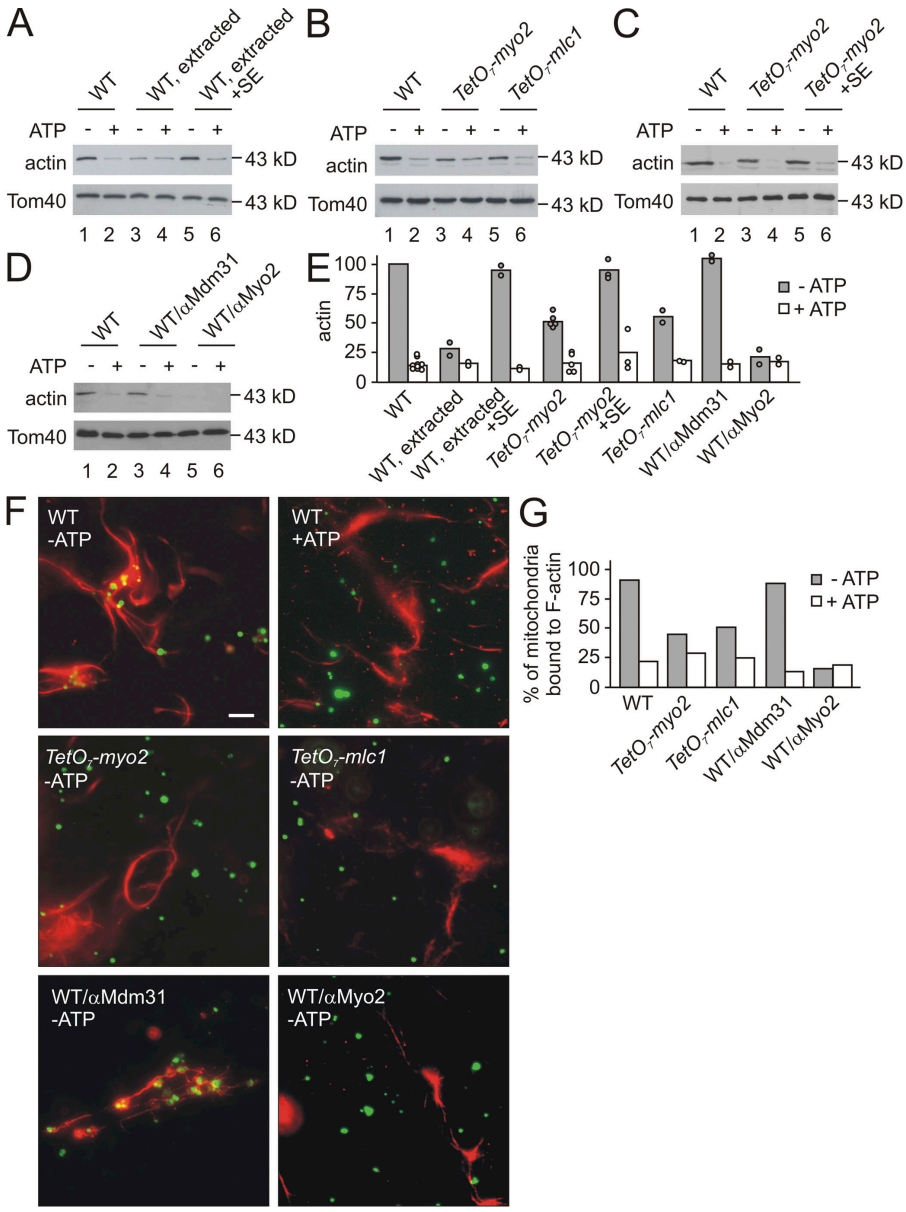

Figure 2. **Myo2 is required for interaction of mitochondria with actin filaments in vitro.** (A) Wild-type (WT) mitochondria were incubated with isolated actin filaments in the absence or presence of ATP. After centrifugation of mitochondria through a sucrose cushion, bound actin was detected by immunoblotting. The mitochondrial protein Tom40 served as a loading control. Lanes 1 and 2 show standard conditions; lanes 3 and 4 show mitochondria that were extracted with 1 M KCl before incubation with actin filaments; lanes 5 and 6 show salt-extracted mitochondria that were incubated with salt extract (SE) before incubation with actin filaments. (B) WT, *TetO7-myo2*, and *TetO7-mlc1* cells were precultured in glucose-containing medium and incubated for 15 h in the presence of 30 µg/ml Dox before isolation of mitochondria. Binding of actin to mitochondria was analyzed under standard conditions in A. (C) Binding of actin to wild-type and *TetO7-myo2* mitochondria was analyzed as in A. Lanes 1 and 2 show wild-type mitochondria analyzed under standard conditions; lanes 3 and 4 show *TetO7-myo2* mitochondria analyzed under standard conditions; lanes 5 and 6 show *TetO7-myo2* mitochondria that were incubated with wild-type SE before incubation with actin filaments. (D) Binding of actin to wild-type mitochondria was analyzed as in A. Lanes 1 and 2 show standard conditions; lanes 3 and 4 show mitochondria preincubated with affinity-purified Mdm31 antibodies at a concentration of 120 ng per mg of mitochondrial protein; lanes 5 and 6 show mitochondria preincubated with the same amount of affinity-purified Myo2 antibodies. (E) Replicate actin sedimentation experiments were quantified by densitometry of actin immunoblots. In each experiment, the signal obtained with wild-type mitochondria in the absence of ATP was set to 100. Bars represent mean values; circles represent individual data points of all measurements that have been performed. Arbitrary units are indicated. (F) Mitochondria isolated from mtGFP-expressing cells were incubated with Alexa Fluor 568–labeled actin filaments and observed by fluorescence microscopy. Depletion of Myo2 and Mlc1 and antibody pretreatments were performed as in A–E. Displayed are merged images of GFP and Alexa Fluor 568 fluorescence. An image of a sample incubated in the presence of ATP is only shown for wild-type mitochondria. Bar, 5 µm. (G) The graph shows a quantification of the experiment in Fig. 2 E. 200 mitochondria were scored per sample.

amino acid substitutions in the secretory vesicle binding site (Pashkova et al., 2006). Vacuole movement is fully functional in these mutants; however, they show severe growth defects because of defective vesicular transport (Pashkova et al., 2006).

Subdomain-specific *myo2* mutants expressing mtGFP were incubated at 30 and 37°C, and mitochondrial morphology was observed by fluorescence microscopy. Wild-type cells and secretion-specific mutants contained branched tubular mitochondria in 84–99% of the cells at both temperatures (Fig. 3, A and B; and Table I). Virtually all budded cells contained mitochondria partitioned to the daughter cell (Table I). In contrast, a large percentage of cells contained aggregated and/or unpartitioned mitochondria in the following six vacuole-specific mutants: *myo2(Q1233R)*, *myo2(D1297G)*, *myo2(L1301P)*, *myo2(N1304S)*, *myo2(N1304D)*, and *myo2(N1307D)* (Fig. 3, A and B; and Table I). Mitochondrial defects were most pronounced in *myo2(L1301P)* and *myo2(Q1233R)*; at a nonpermissive temperature, 83 or 73%, respectively, of mutant cells contained mitochondria that were aggregated or clumped in the mother cell, whereas buds were largely devoid of mitochondria (Fig. 3, A and B; and Table I). Only two vacuole-specific alleles, *myo2(G1248D)* and *myo2(D1297N)*, did not produce any significant mitochondrial defects. We conclude that the subdomain of the Myo2 tail responsible for vacuolar movement is also important for mitochondrial distribution and morphology.

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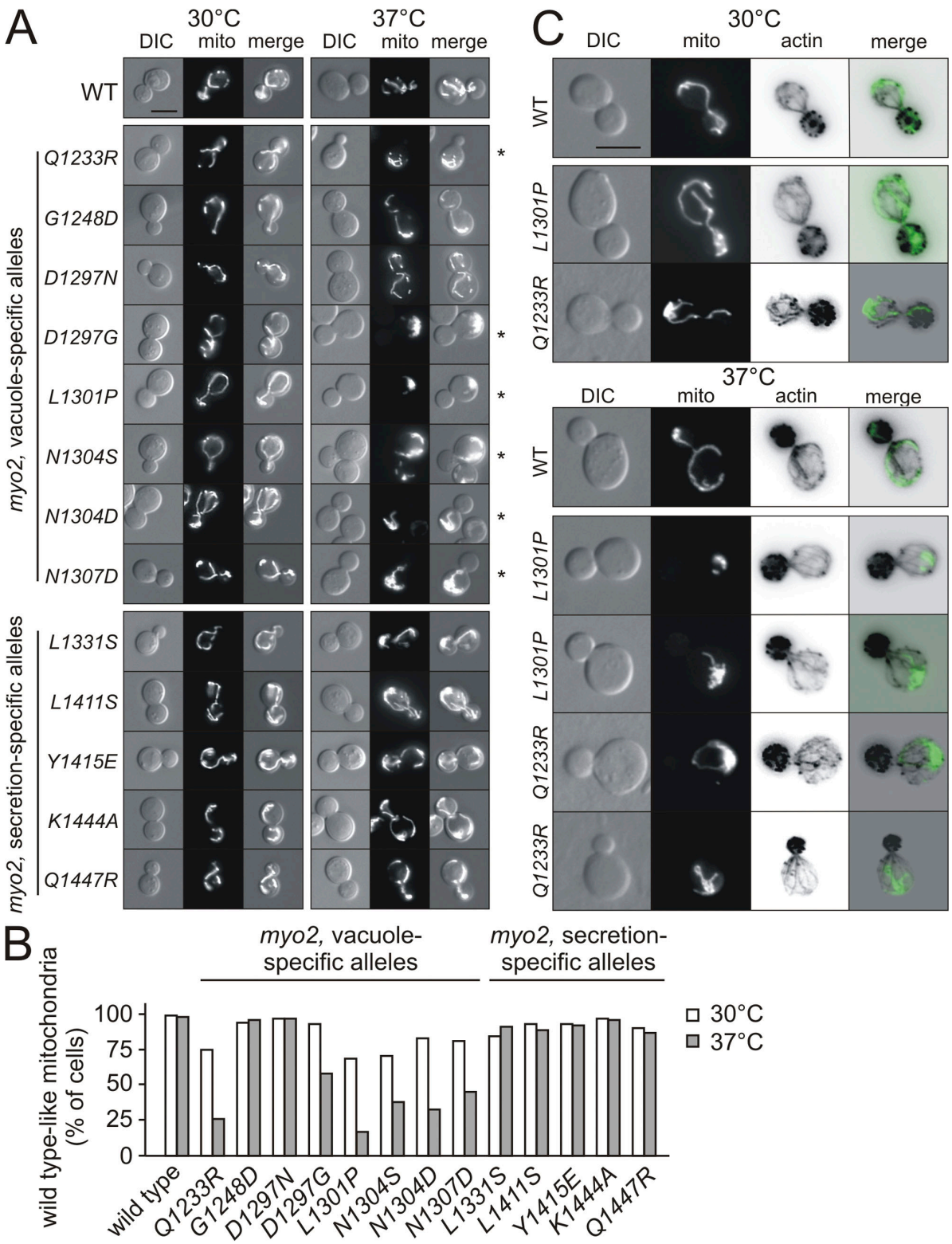

Figure 3. **Specific mutations in the Myo2 cargo-binding domain produce mitochondrial morphology and inheritance defects.** (A) Wild-type (WT) and *myo2* mutant cells expressing mtGFP were cultured in glucose-containing medium overnight at 30°C. Cultures were then diluted with fresh medium and either kept at 30°C (left) or shifted to 37°C for 3 h (right). Cells were analyzed by DIC and fluorescence microscopy. Asterisks indicate characteristic mitochondrial partitioning defects. Bar, 5 µm. (B) The graph shows a quantification of mitochondrial phenotypes scored in the experiment shown in A. Displayed is an excerpt of data presented in Table I. (C) Wild-type, *myo2-L1301P*, and *myo2-Q1233R* cells were grown as in A and analyzed as in Fig. 1 B. Bar, 5 µm.

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Table I. **Mitochondrial distribution and morphology in *myo2* mutants**

Strain	Temperature	Mitochondrial distribution and morphology				
		Wild type–like	Aggregated or fragmented	Aggregated in mother, bud empty	Tubular in mother, bud empty	No. of cells scored
	°C	% of cells	% of cells	% of cells	% of cells	
Wild type	30	99	0	0	1	300
	37	98	0	0	2	300
Alleles producing vacuolar transport defects						
myo2(Q1233R)	30	75	0	12	13	300
	37	26	1	45	28	300
myo2(G1248D)	30	94	0	0	6	300
	37	96	0	0	4	300
myo2(D1297N)	30	97	0	0	3	300
	37	97	1	1	1	300
myo2(D1297G)	30	93	0	2	5	300
	37	58	2	32	8	300
myo2(L1301P)	30	69	3	15	13	300
	37	17	0	52	31	300
myo2(N1304S)	30	70	2	8	20	300
	37	38	1	42	19	300
myo2(N1304D)	30	83	1	11	15	300
	37	33	0	44	23	300
myo2(N1307D)	30	81	1	4	14	300
	37	45	0	30	25	300
Alleles producing vesicular transport defects						
myo2(L1331S)	30	84	16	0	0	100
	37	91	9	0	0	100
myo2(L1411S)	30	93	7	0	0	100
	37	89	11	0	0	100
myo2(Y1415E)	30	93	7	0	0	100
	37	92	8	0	0	100
myo2(K1444A)	30	97	3	0	0	100
	37	96	4	0	0	100
myo2(Q1447R)	30	90	10	0	0	100
	37	87	13	0	0	100

The fact that two vacuole-specific alleles did not produce mitochondrial defects suggests that the mitochondrial binding site on the Myo2 tail largely overlaps with, but is not identical to, the vacuolar binding site.

In further experiments, we concentrated on mutants *myo2(L1301P)* and *myo2(Q1233R)*, as these alleles produced the clearest mitochondrial phenotypes. To exclude the possibility that mitochondrial aggregation is caused by defects in the organization of the actin cytoskeleton, mutant and wild-type cells expressing mtGFP were incubated at 30 or 37°C, cells were fixed, microfilaments were stained with rhodamine phalloidin, and mitochondria and the actin cytoskeleton were observed by fluorescence microscopy. Again, mitochondria formed aggregates in *myo2(L1301P)* and *myo2(Q1233R)* mutants at 37°C, and buds were devoid of mitochondria. However, the actin cytoskeleton appeared normal under all conditions (Fig. 3 C). We conclude that specific mutations in the proximal half of the Myo2 globular tail affect mitochondrial distribution and morphology.

The globular tail of Myo2 is required for binding of mitochondria to actin filaments in vitro

We asked whether the globular tail of Myo2 is critical for interaction of mitochondria with actin filaments. Mitochondria were isolated from the wild type and *myo2(L1301P)* and *myo2(Q1233R)* mutants, incubated with actin filaments in the absence or presence of ATP, and centrifuged through a sucrose cushion, and bound actin was quantified by immunoblotting. We observed that actin-binding activity of mutant mitochondria was reduced to 61 or 37%, respectively, in comparison to the wild type (Fig. 4, A and B). Similar effects were found when mitochondrial actin-binding activity was observed directly in the visual assay. While in the absence of ATP, 90% of wild-type mitochondria were found associated with actin filaments, and this number was reduced to 34% for *myo2(L1301P)* mitochondria and 36% for *myo2(Q1233R)* mitochondria (Fig. 4, C and D). These results point to an important role of the proximal half of the Myo2 globular tail in mediating mitochondria–cytoskeleton interactions.

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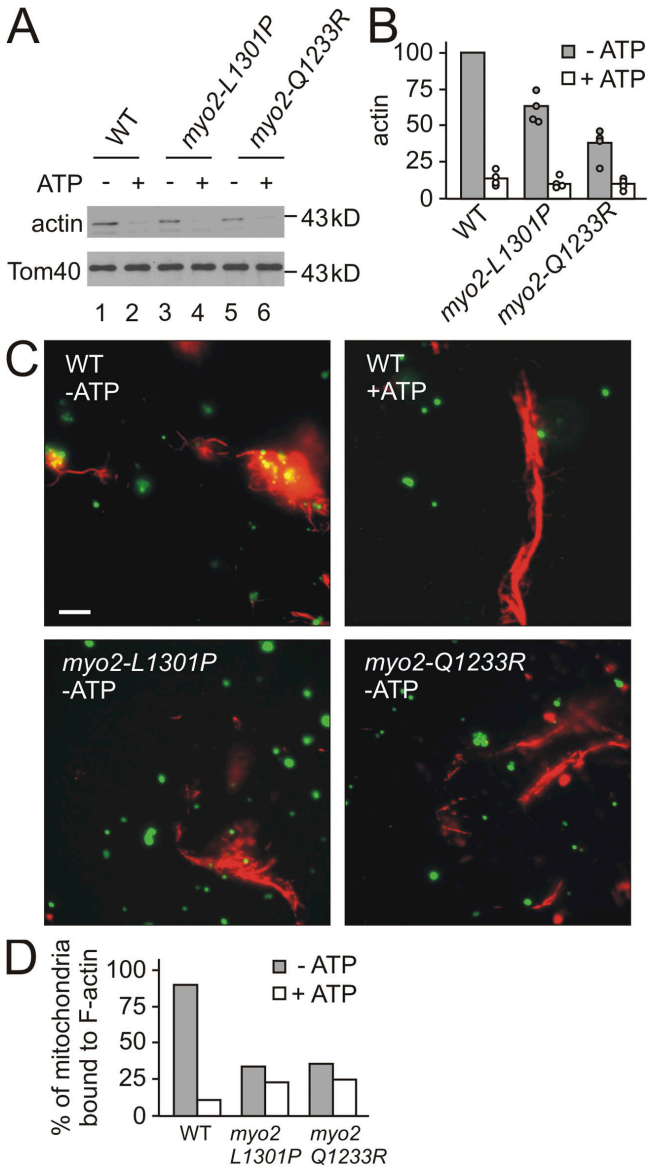

Figure 4. **Mutations in the cargo-binding domain of Myo2 impair binding of mitochondria to actin filaments in vitro.** (A) Wild-type (WT), *myo2-L1301P*, and *myo2-Q1233R* cultures were grown in glucose-containing minimal medium at 30°C and shifted to 37°C for 3 h before isolation of mitochondria. Binding of actin filaments to mitochondria under standard conditions was analyzed as in Fig. 2 A. (B) Replicate actin sedimentation experiments were quantified as in Fig. 2 E. Bars represent mean values; circles represent individual data points of all measurements that have been performed. (C) Cultures of mtGFP-expressing cells were grown as in A and the binding of mitochondria to actin filaments was analyzed as in Fig. 2 F. An image of a sample analyzed in the presence of ATP is shown only for wild-type mitochondria. Bar, 5 µm. (D) The graph shows a quantification of the experiment in Fig. 4 C. 200 mitochondria were scored per sample.

Myo2 is required for directed movement of mitochondria into the bud

Next, we investigated whether Myo2 is required for anterograde movement of mitochondria from the mother cell into the bud. Logarithmically growing wild-type and *myo2(L1301P)* cells expressing mtGFP were shifted to 37°C for 3 h and then observed by time-lapse live cell microscopy for 30 min at ambient temperature. 3D data stacks of budded cells were obtained every 3 min by confocal microscopy. In wild-type cells, mitochondria were observed to move around and undergo frequent shape changes by fusion and fission. Importantly, the organelles were well partitioned in mother and daughter cells, both in cells carrying large and small buds (Fig. 5 A). In contrast, movement of mitochondria was restricted to a much smaller area in *myo2(L1301P)* cells, and even large buds were often found to be devoid of mitochondria (Fig. 5 A). Close inspection of data stacks obtained from wild-type cells ($n = 5$) revealed that an average of 6.4 mitochondria per hour passed the bud neck. However, in *myo2(L1301P)* cells ($n = 11$), only 0.36 mitochondria per hour entered the bud. These data demonstrate that anterograde, bud-directed mitochondrial movement is severely impaired by mutation of the cargo binding site of Myo2.

To corroborate these findings, we quantified the number of buds lacking mitochondria in *myo2* mutants. Wild-type cells and cells with secretion-specific *myo2* alleles carried buds devoid of mitochondria at a frequency of 2–6% (Fig. 5 B). In accordance with the mitochondrial morphology defects described above (compare Fig. 3 A and Table I), six *myo2* strains defective in vacuolar inheritance produced significant mitochondrial partitioning defects (Fig. 5 B). Again, these phenotypes were most pronounced in *myo2(L1301P)* and *myo2(Q1233R)* mutants, which displayed buds devoid of mitochondria in 78 or 72% of cells at 37°C, respectively. We conclude that Myo2-mediated anterograde movement of mitochondria is important for mitochondrial inheritance during budding of yeast cells.

Discussion

Several lines of evidence support a direct role of the class V myosin, Myo2, as a mediator of mitochondrial motility in *S. cerevisiae*. Depletion of Myo2 or mutation of its cargo-binding domain produces severe mitochondrial morphology and inheritance defects in cells containing an apparently normal actin cytoskeleton. In particular, cells carrying specific *myo2* mutant alleles show pronounced mitochondrial defects that are similar to vacuolar inheritance defects reported in the literature (Pashkova et al., 2006). The fact that entry of mitochondria into the bud is largely blocked in these mutants strongly argues against an indirect role of Myo2 as a transporter of mitochondrial retention factors because these would become effective only after mitochondria have reached the bud tip. Notably, mitochondria lacking functional Myo2 are defective in ATP-sensitive binding to actin filaments in vitro. A critical role of Myo2 on the mitochondrial surface is demonstrated by the following two observations: actin-binding activity of Myo2-depleted mitochondria can be restored by the addition of salt extracts prepared from wild-type mitochondria; and interactions of wild-type mitochondria with actin filaments can be blocked completely with antibodies directed against Myo2. In summary, these results demonstrate that Myo2 is of major importance for mitochondrial movement in yeast.

A key role of Myo2 in mitochondrial transport is compatible with several observations that have been reported early after the discovery of the actin dependence of mitochondrial inheritance. Yeast actin mutants that exhibit specific mitochondrial defects contain amino acid exchanges under or near the myosin

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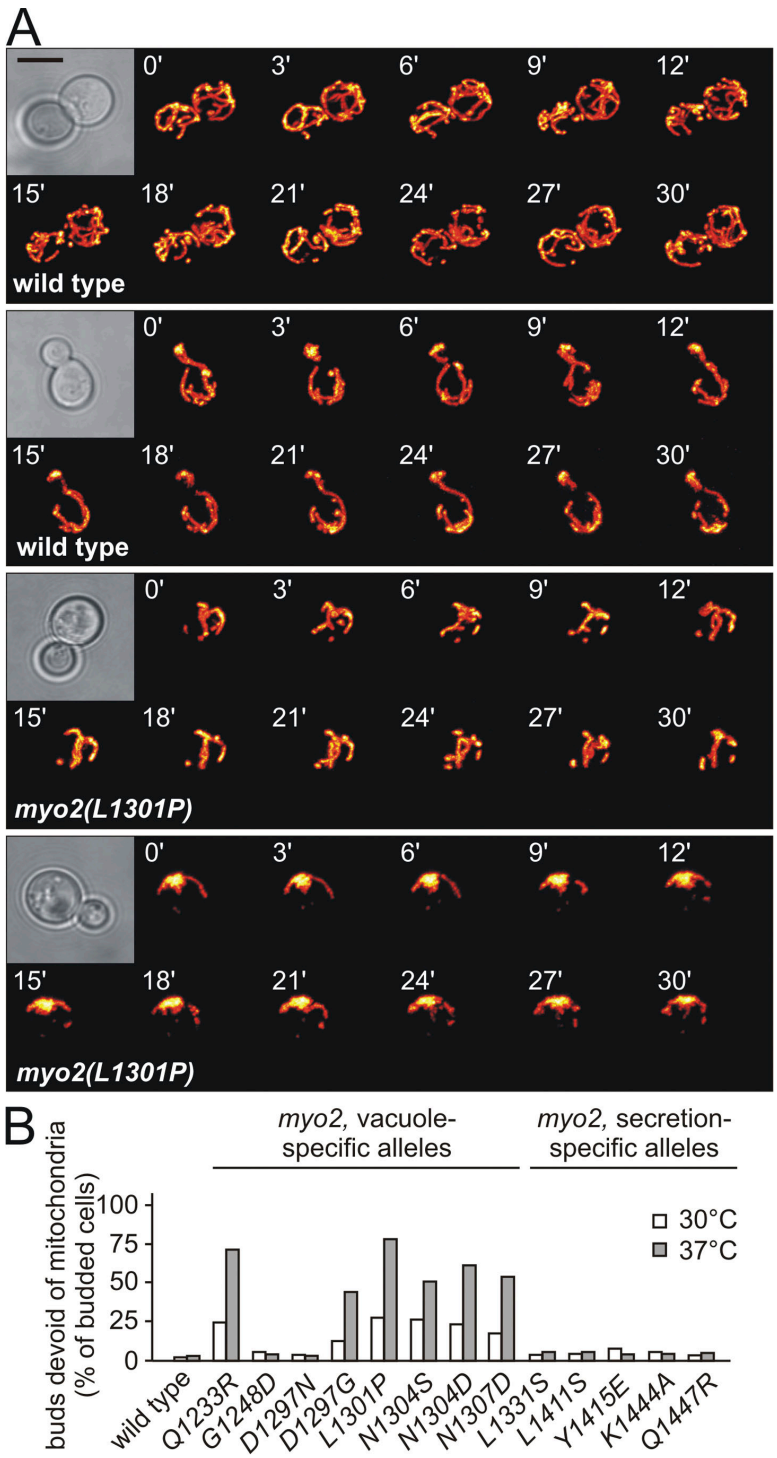

Figure 5. **Mutations in the cargo-binding domain of Myo2 impair anterograde mitochondrial movement and partitioning of mitochondria to the bud.** (A) Wild-type and *myo2-L1301P* cells expressing mtGFP were precultured in glucose-containing minimal medium at 30°C, shifted to 37°C for 3 h, and observed by confocal time-lapse microscopy at ambient temperature. The first image of each series is a bright-field image; mitochondria are shown as maximum intensity projections of several optical planes. One representative cell carrying a large bud and one representative cell carrying a small bud are shown for the wild type and *myo2(L1301P)*. Time is indicated in minutes. Bar, 5 µm. (B) Wild-type and *myo2* mutant cells expressing mtGFP were grown as in Fig. 3 A and buds were scored for the presence of mitochondria. 200 budded cells were analyzed for each strain at each temperature.

footprint (Drubin et al., 1993). This observation suggested immediately that actin–myosin interactions might underlie the cytoplasmic organization of mitochondria (Drubin et al., 1993). Furthermore, saturation of myosin-binding sites on actin filaments with the globular head domain of skeletal muscle myosin was observed to block binding of mitochondria in vitro (Lazzarino et al., 1994), and a mitochondria-associated motor activity was found to display an ATP concentration dependence similar to that of myosin family members in a microfilament sliding assay (Simon et al., 1995). However, it has been argued that Myo2 is unlikely to be the major mitochondrial motor because reduction of the length of the Myo2 lever arm in the *myo2-Δ6IQ* mutant is expected to decrease the velocity of the motor but was found to have no effect on the mean velocity of mitochondrial movement (Boldogh et al., 2004). However, it has been pointed out that Myo2-dependent movement of secretory vesicles in the *myo2-Δ6IQ* mutant is even faster than that of mitochondria in wild-type cells, which suggests that, in the case of mitochondria, Myo2 velocity may not be the limiting factor (Frederick and Shaw, 2007). We consider it likely that several motor molecules must cooperate

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to move a large organelle, such as a mitochondrion. In this case, velocity would not be expected to be limited by the speed of a single, processively moving motor molecule.

The fact that mutations in the proximal half of the Myo2 globular tail impair binding of mitochondria to actin filaments suggests that the sites responsible for establishing interactions of the motor with mitochondria and vacuoles overlap. Although the identity of the mitochondrial Myo2 receptor remains to be identified, two previously characterized proteins potentially cooperate with Myo2 in mitochondrial distribution and morphology. Ypt11 is a small Rab-type GTPase, and Mmr1 is a protein concentrated on mitochondria in the bud. Both components act as high-dose suppressors of *myo2* mutants, and a Δ*ypt11* Δ*mmr1* double mutant shows severe mitochondrial partitioning defects (Itoh et al., 2002, 2004). However, Δ*ypt11* and Δ*mmr1* single mutants do not display strong mitochondrial phenotypes (Dimmer et al., 2002; Itoh et al., 2002, 2004), indicating that neither protein on its own is sufficient to act as a mitochondrial Myo2 receptor. Moreover, it has not been demonstrated whether Ypt11 and Mmr1 are directly required for interaction of mitochondria with the cytoskeleton. As Ypt11 does not seem to associate with mitochondria (Itoh et al., 2002, 2004), it appears that additional, yet unknown proteins are involved in anchoring Myo2 to the mitochondrial surface.

Boldogh et al. (2001a) have proposed an alternative mechanism to explain mitochondrial motility in budding yeast. They found that subunits of the Arp2/3 complex, the cell's most important initiator of actin polymerization, are located on the mitochondrial surface and that mitochondrial motility is impaired when actin dynamics are perturbed. These observations raised the possibility that mitochondria might move by an actin polymerization–dependent mechanism similar to the intracellular movement of certain bacterial pathogens, such as *Listeria monocytogenes* (Boldogh et al., 2001a, 2006). However, actin-dependent movement of mitochondria differs from other actin polymerization–dependent motility processes in an important aspect: mitochondrial movement occurs along preexisting actin cables (Fehrenbacher et al., 2004), whereas intracellular bacterial pathogens and endocytic membranes are propelled by growth of a newly generated comet-like actin tail (Kaksonen et al., 2006; Stevens et al., 2006). Moreover, it is difficult to reconcile this model with our observations that Myo2 is required for interaction of mitochondria with actin filaments in vitro, and that anterograde mitochondrial movement and entry into the bud is impaired in cargo binding–defective *myo2* mutants in vivo. Thus, we consider it unlikely that Arp2/3 complex–dependent actin polymerization is the main mechanism mediating mitochondrial motility in yeast. Recent evidence suggests that somewhat redundant mechanisms for mitochondrial movement have evolved in fungi. For example, microtubule-dependent transport of mitochondria in the filamentous fungus *Neurospora crassa* is mediated by an evolutionarily conserved kinesin-related motor protein. When this motor is lacking, expression of an unconventional fungi-specific kinesin that replaces its function is induced (Fuchs and Westermann, 2005). In analogy, we consider it possible that Myo2 is the major transporter of mitochondria in yeast, and actin polymerization–driven motility might contribute to a minor extent.

Our work establishes for the first time a direct role of Myo2 as a motor protein moving mitochondria in yeast. Some observations reported in the literature provide evidence for an involvement of myosin-related proteins in mitochondrial motility in other organisms also. A putative unconventional myosin has been detected on motile mitochondria in locust photoreceptors (Stürmer and Baumann, 1998), partitioning of mitochondria and other organelles during spermatogenesis is defective in *Caenorhabditis elegans* class VI myosin mutants (Kelleher et al., 2000), and a plant-specific class XI myosin was found to colocalize with mitochondria and chloroplasts in maize cells (Wang and Pesacreta, 2004). Interestingly, cellular fractionation experiments and immunoelectron microscopy revealed that a class V myosin encoded by the *dilute* gene is associated with mitochondria in mammalian melanoma cells (Nascimento et al., 1997). Thus, it will be interesting to see in the future whether yeast and mammalian cells use similar mechanisms to mediate actin-dependent mitochondrial motility.

Materials and methods

Yeast strains

Growth and manipulation of yeast strains was performed according to standard procedures (Burke et al., 2000). All strains used in this study are derivatives of BY4741, BY4742, or BY4743 (Brachmann et al., 1998). *TetO*$_7$ promoter strains (Mnaimneh et al., 2004) and isogenic wild-type R1158 were obtained from BioCat. Plasmid BG1805 containing the *MYO2* gene under control of the *GAL1* promoter was obtained from BioCat and transformed into strain BY4742. To construct yeast strains expressing *myo2* mutant alleles, a heterozygous *myo2* deletion strain (Giaever et al., 2002) was obtained from EUROSCARF and transformed with plasmid pRS416-MYO2 (Catlett et al., 2000). The resulting strain was sporulated, tetrads were dissected, and a haploid strain was isolated that contained a genomic *myo2::kanMX4* deletion allele and the *MYO2* wild-type allele on the plasmid. This strain served as a recipient for plasmid pRS413-MYO2 (Catlett and Weisman, 1998) to construct the wild-type control and for pRS413-MYO2–based plasmids containing *myo2* mutant alleles (Catlett and Weisman, 1998; Catlett et al., 2000; Pashkova et al., 2006). After counterselection against pRS416-MYO2 by growth on 5-fluoroorotic acid–containing medium, strains were obtained that expressed *myo2* alleles from single copy plasmids under control of the endogenous *MYO2* promoter.

Staining of cellular structures

To visualize mitochondria, yeast strains were transformed with plasmid pYX142-mtGFP (Westermann and Neupert, 2000). mtDNA nucleoids were stained in methanol-fixed cells according to published procedures (Jones and Fangman, 1992). The actin cytoskeleton was stained with rhodamine phalloidin (Invitrogen) as described previously (Amberg, 1998).

Microscopy

Electron microscopy of yeast cells (Dürr et al., 2006) and isolated mitochondria (Meeusen et al., 2004) was performed as described previously. Differential interference contrast (DIC) and epifluorescence microscopy was performed using a microscope (Axioplan 2; Carl Zeiss, Inc.) equipped with a Plan-Neofluar 100× 1.30 NA Ph3 oil objective (Carl Zeiss, Inc.). Samples were embedded in 1% low-melting-point agarose and observed at room temperature. Images were recorded with a monochrome camera (Evolution VF Mono Cooled; Intas Science Imaging Instruments GmbH) and processed with Image Pro Plus 5.0 and Scope Pro 4.5 software (Media Cybernetics, Inc.). CorelDRAW graphics suite version 12.0 (Corel Corporation) was used for mounting of the figures; image manipulations other than minor adjustments of brightness and contrast were not performed.

For time-lapse live cell microscopy, cells were grown in glucose-containing minimal medium to early logarithmic growth phase and transferred to a percolation chamber that was constantly flushed with fresh medium and kept at ambient temperature. Cells were mounted in 1% low-melting-point agarose to inhibit spatial movements of the cells. For image acquisition, a beam-scanning confocal microscope (TCS SP2; Leica) equipped

with a Plan-apo 63× 1.2 NA water immersion lens (Leica) was used. Images were averaged fourfold. Displayed are maximal intensity projections of 3D data stacks. Except smoothing and contrast stretching using the image acquisition software Imspector (Max-Planck-Innovation GmbH), no further image processing was applied.

Analysis of mitochondria–actin interactions in vitro

Phalloidin-stabilized actin filaments were prepared by polymerizing nonmuscular human actin (Tebu-Bio GmbH) according to the manufacturer's instructions. To fluorescently label actin filaments, 15 µg of Alexa Fluor 568–labeled actin (Invitrogen) was mixed with 100 µg of nonlabeled actin before polymerization.

For actin/mitochondria cosedimentation, isolated mitochondria were further purified by sucrose density gradient centrifugation as described previously (Altmann et al., 2007). The purity of representative mitochondria preparations was checked by electron microscopy (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200709099/DC1). Preparation of salt-washed mitochondria and mitochondrial salt extracts (Boldogh et al., 1998), binding of filamentous actin (at a concentration of 100 µg/ml) to purified mitochondria, and cosedimentation of actin with mitochondria (Lazzarino et al., 1994) were performed according to published procedures. Affinity-purified Myo2 antibodies used for pretreatment of isolated mitochondria were a gift from L.S. Weisman (University of Michigan, Ann Arbor, MI). Actin was detected by immunoblotting with monoclonal PanActin Ab-5 antibodies (Thermo Fisher Scientific), and mitochondria were detected with polyclonal Tom40 antibodies (a gift from D. Rapaport, Universität Tübingen, Tübingen, Germany). ECL-generated bands were quantified by densitometry using Scion Image software (Scion Corporation).

For visualization of actin–mitochondria interactions, 200 µg of mitochondria isolated from strains expressing mtGFP were incubated with 2.25 µg of fluorescently labeled actin filaments in 50 µl RM buffer (0.6 M sorbitol, 20 mM Hepes/KOH, pH 7.4, 2 mM $MgCl_2$, 0.1 M KCl, 1 mg/ml of fatty acid–free bovine serum albumin, protease inhibitor cocktail, and 1 mM PMSF). To assay ATP sensitivity, either 50 U/ml apyrase (−ATP) or 2 mM ATP, 0.1 mg/ml creatine kinase, and 10 mM creatine phosphate (+ATP) were added. Samples were incubated for 10 min at 30°C, embedded in low-melting-point agarose, and observed by fluorescence microscopy.

Online supplemental material

Fig. S1 shows cross sections of complete wild-type and *TetO7-myo2* cells analyzed by electron microscopy. Fig. S2 shows preparations of purified wild-type and *TetO7-myo2* mitochondria analyzed by electron microscopy. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200709099/DC1.

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